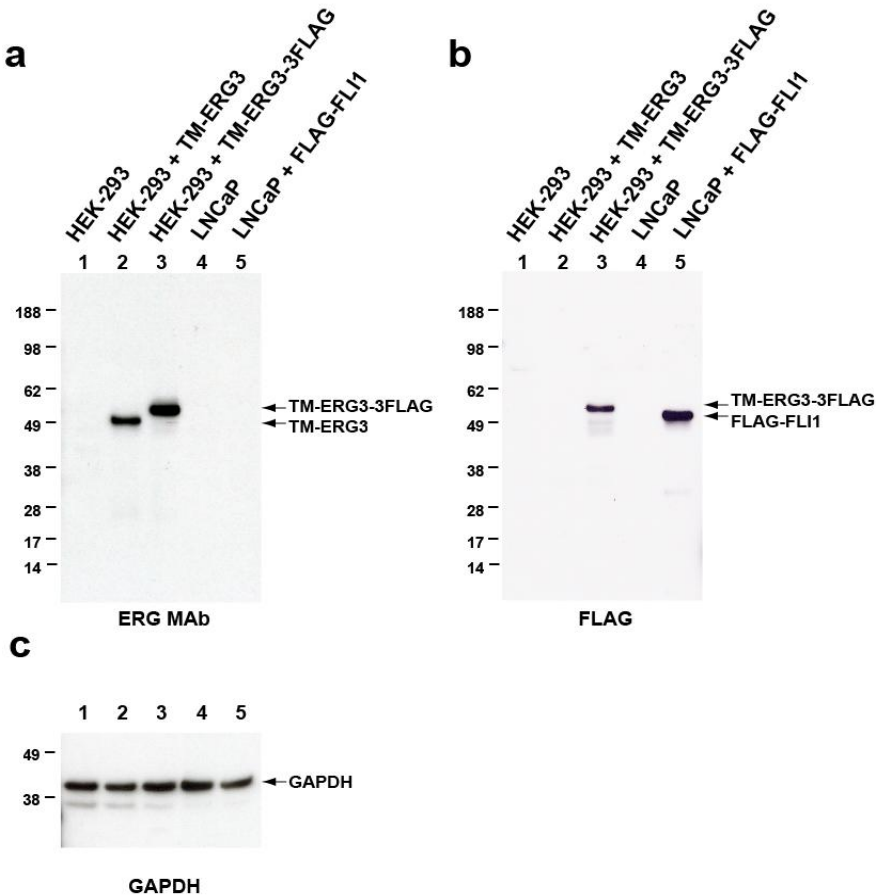# Supplementary materials

## Supplementary results

Supplementary Figure 1

Supplementary Figure 1. Demonstration of the specificity of the ERG-MAb for selective recognition of ERG protein in comparison to closely related FLI1. HEK-293 cells were transiently transfected with pCMV control vector (lane 1), pCMV-*TMPRSS2-ERG3* (lane 2) vector and pCMV-*TMPRSS2-ERG3-FLAG* vector (lane 3). LNCaP cells were infected with 10 pfu each of control AdEasy adenovirus vector (lane 4) or with FLAG-FLI1 adenovirus vector (lane 5) kindly provided by Dr. Dennis K. Watson [1]. Total cell lysates equivalent to 8 µg (lanes 1 to 3) or 4 µg (lane 4 and 5) of proteins were separated on NuPAGE Bis-Tris (4-12%) gels and analyzed for the ERG protein by Western blot by using the ERG-MAb. (a) ERG-MAb recognized both un-tagged (lane 2) and FLAG-*TMPRSS2-ERG3* (lane 3). (b) Polyclonal anti-FLAG antibody recognized ERG3-FLAG (lane 3) and FLAG-FLI1 (lane 4) proteins. (c) Membrane was stripped and re-blotted with anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody as the input control. Expression vectors for lane 1 and 2 are same as for Figure S1. pCMV-*TMPRSS2-ERG3* vector used in lane 2 was modified with 3XFLAG tag at the ERG3 C-terminus for lane 3.

Supplementary Figure 2
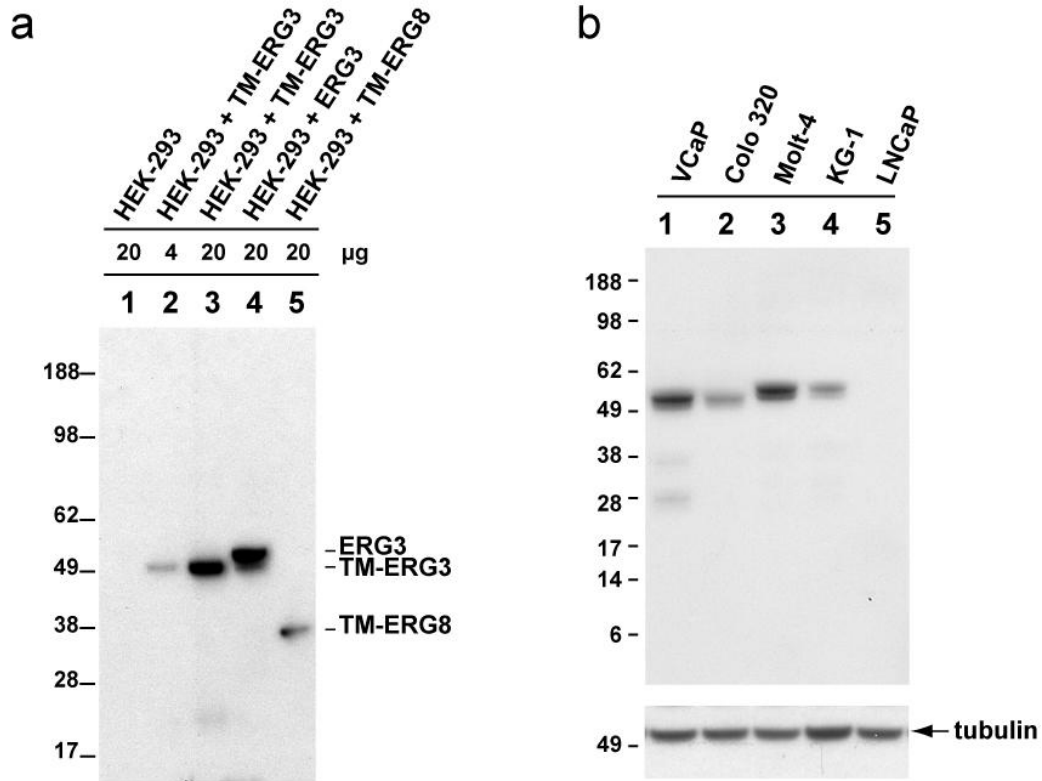
Supplementary Figure 2. Detection of ectopic and endogenous ERG protein in cells lines. (a) Expression of ERG protein products encoded by common splice variants of *TMPRSS2-ERG* fusions present in prostate tumors. HEK-293 cells were transiently transfected with pCMV control vector (lane 1), pCMV-*TMPRSS2* (exon 1)-*ERG3* (exons 8-16, GenBank accession number: NM_001136154) vector (lanes 2 and 3), pCMV-*ERG3* (exons 5-16, GenBank accession number*:* NM_001136154) (lane 4) or pCMV-*TMPRSS2* (exon 1)-*ERG8* (exon 8-ERG8 specific sequence, GenBank accession number: AY204742) vector (lane 5) as described before [2]. *ERG* exon numbers correspond to Owczarek *et al.*, report [3]. Total cell lysates equivalent to 4 or 20 µg of proteins were separated on NuPAGE Bis-Tris (4-12%) gels and ERG protein was analyzed by Western blot using the ERG-MAb. ERG-MAb recognized expected sizes of ERG protein products encoded by *TMPRSS2-ERG3* fusion type-A cDNA (lanes 2 and 3), wild type *ERG3* cDNA (lane 4) and *TMPRSS2-ERG8* fusion type-A cDNA (lane 5). (b) Detection of ERG protein in human tumor cell lines. Fifty µg of total cell lysates from prostate cancer (VCaP, lane 1; LNCaP, lane 5), colon cancer (COLO 320, lane 2), acute lymphoblastic leukemia (MOLT4, lane 3) and acute myelogenous leukemia (KG1, lane 4) derived cell lines were separated on NuPAGE Bis-Tris (4-12%) gels. The endogenous ERG protein was assessed by Western blot by using the ERG-MAb. LNCaP cells do not harbor ERG fusion or express ERG protein.
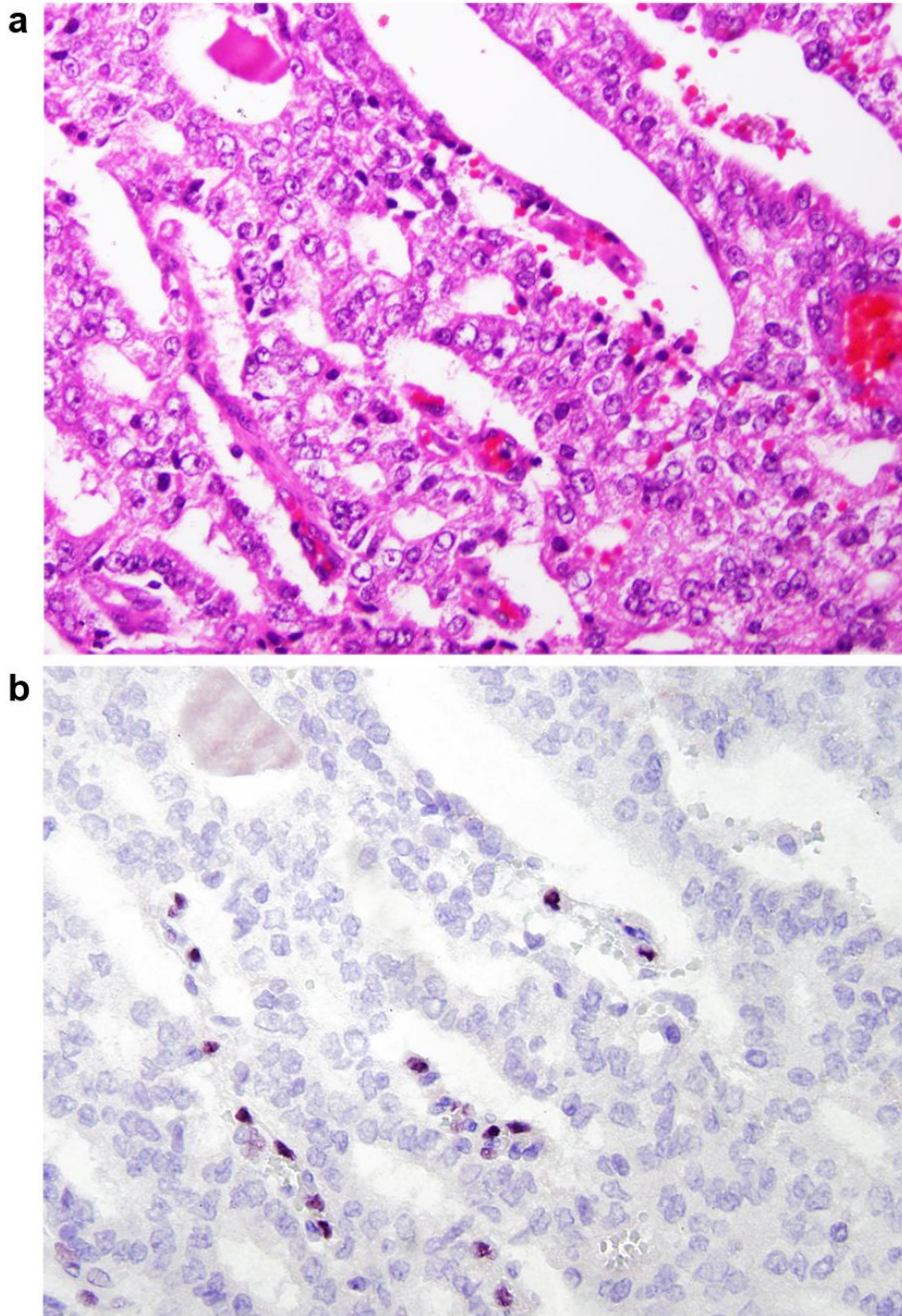
Supplementary Figure 3

Prostatectomy   Lymph node metastasis
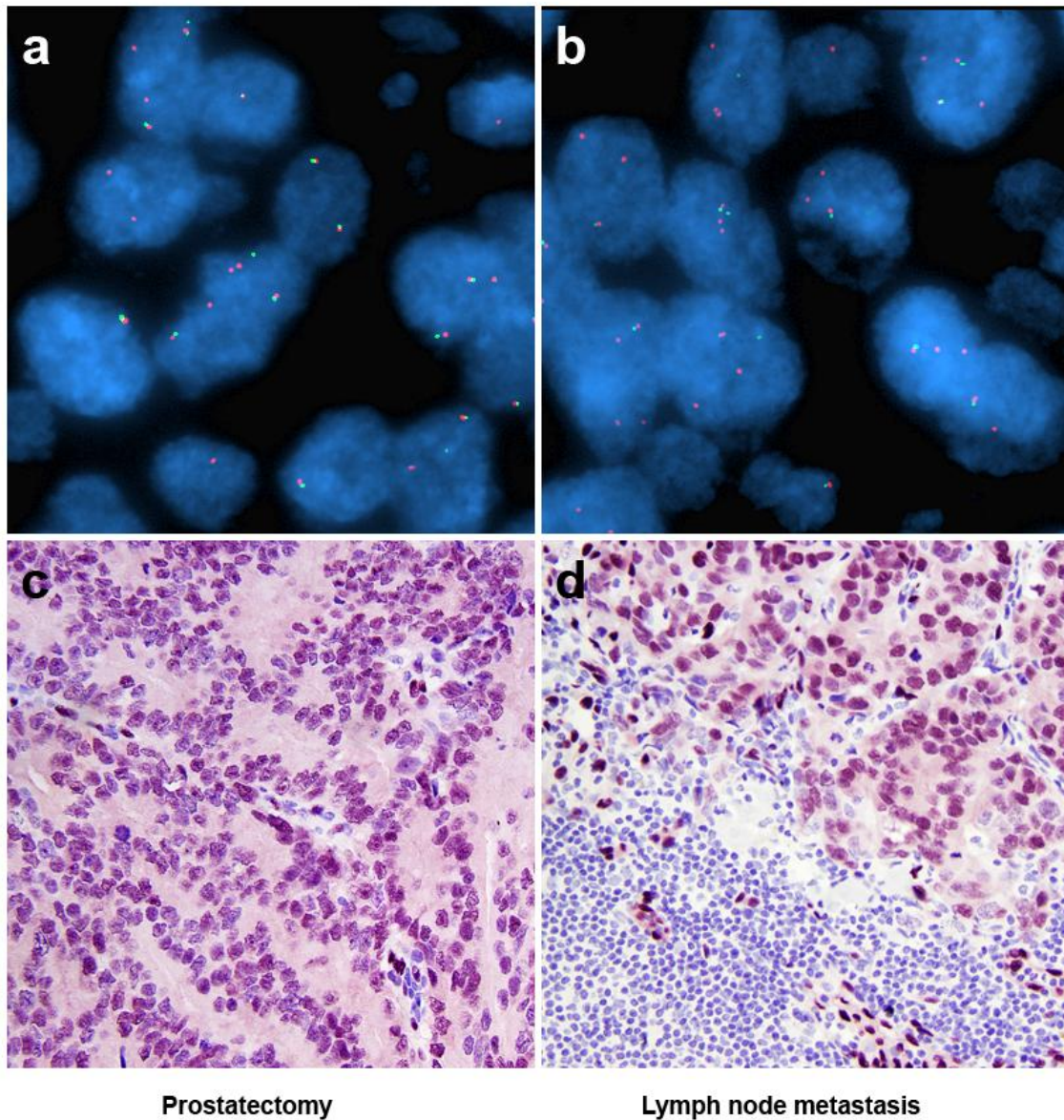
Supplementary Figure 3. Similarity of the *TMPRSS2-ERG* genomic rearrangements in the sections of ERG-MAb positive primary tumor and lymph node metastasis specimens of patients. Three ERG positive cases and one negative case where primary and metastatic tumor specimens were available were examined for *TMPRSS2-ERG* genomic fusion by dual-color interphase FISH. In all of these specimens the FISH results (a and b) were identical to the ERG-MAb status (c and d). Separate and distinct red and green signals indicate a fusion of the *TMPRSS2* and *ERG* loci. Loss of the green signal complementary to the telomeric 5'*ERG* represents a deletion in this chromosomal region. For the non-rearranged *ERG* locus, the set of probes appear either as juxtaposed red and green signals or yellow spots due to the overlap between the red centromeric or green telomeric probes.
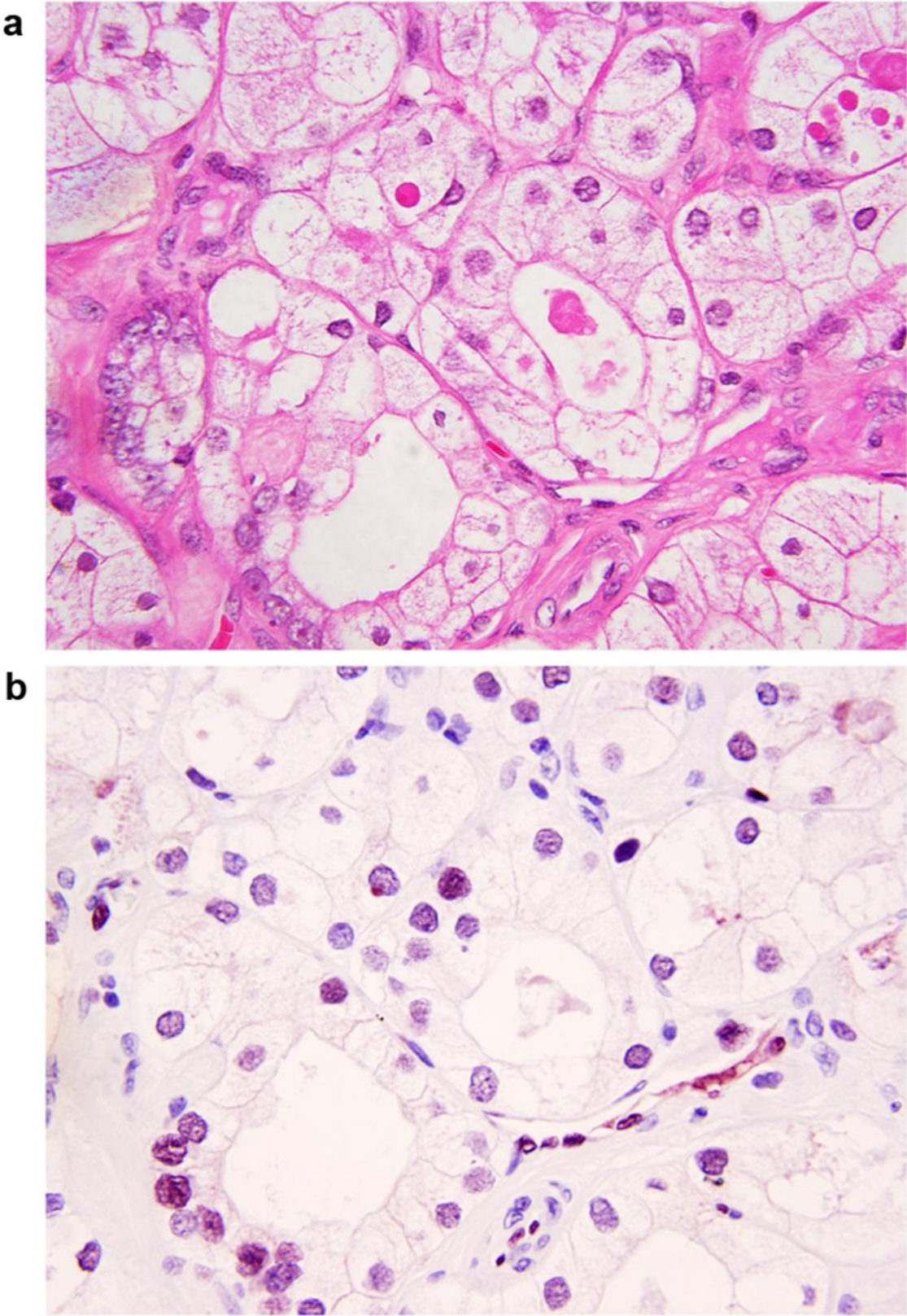
Supplementary Figure 4

Supplementary Figure 4. Distinct morphology of ERG positive endothelial cells in a ERG negative prostate cancer specimen. Poorly differentiated carcinoma/Gleason pattern 4: H&E stain (a) and ERG-MAb IHC (b). The tumor cells have abundant cytoplasm and have a cohesive growth pattern with attempted glandular differentiation. Note, the positive nuclei with linear distribution, can be identified in the H&E stain as endothelial cells associated with erythrocytes.
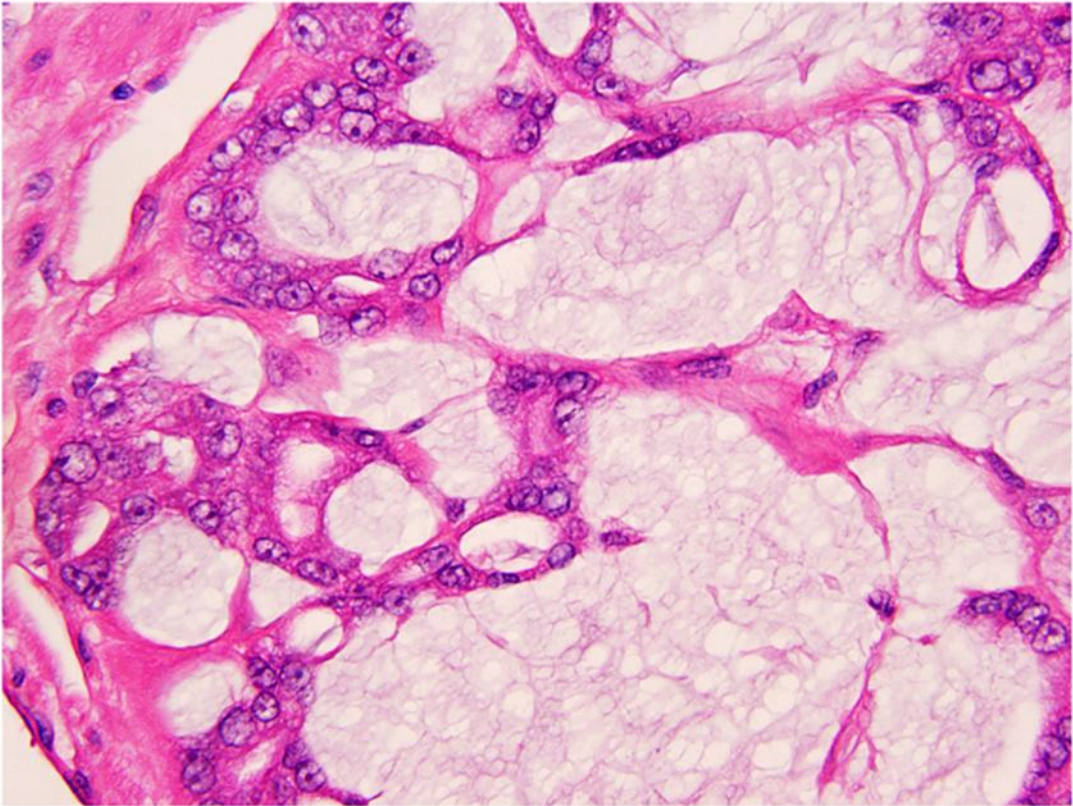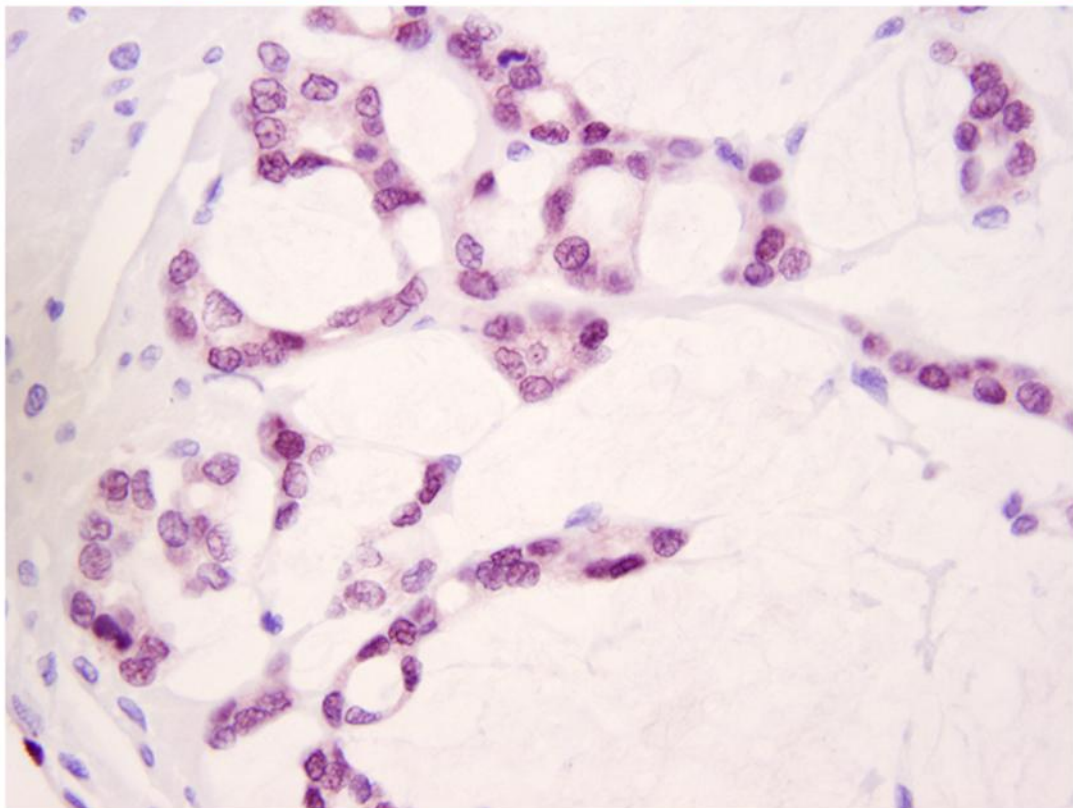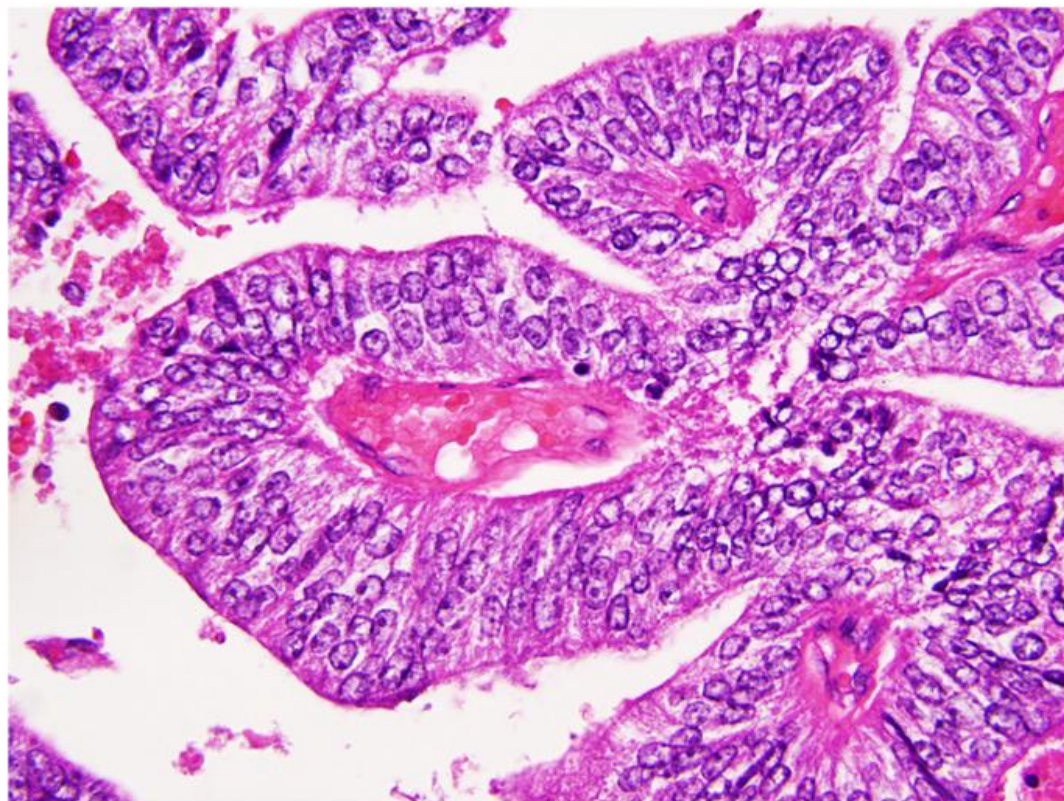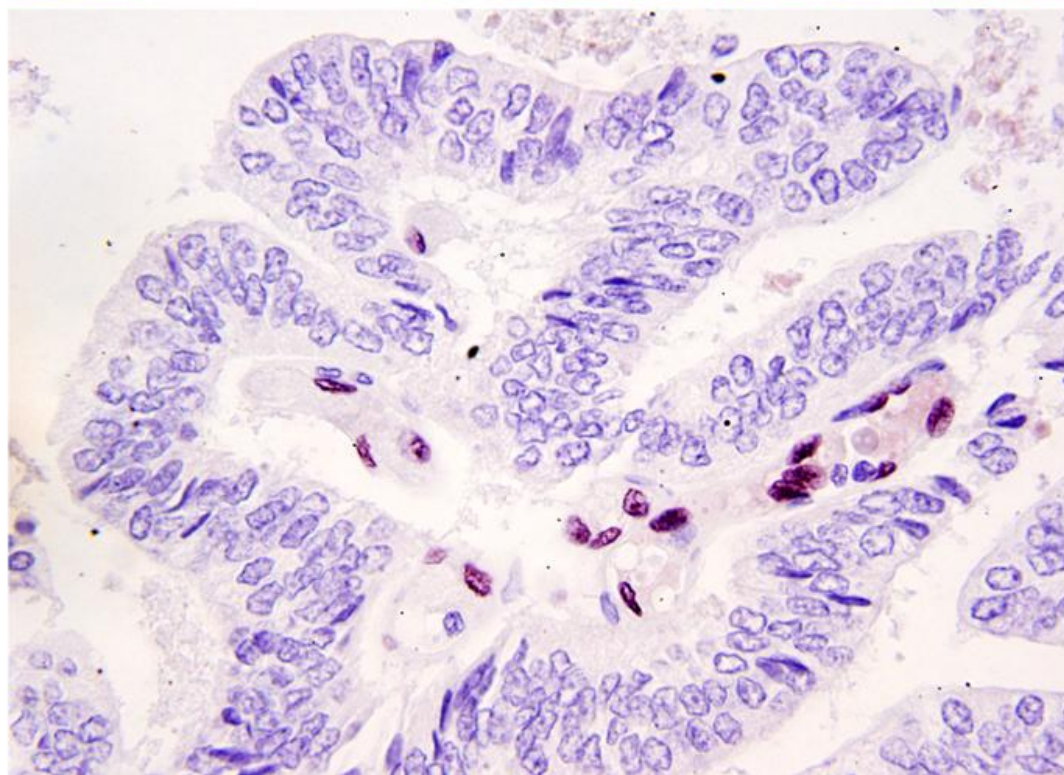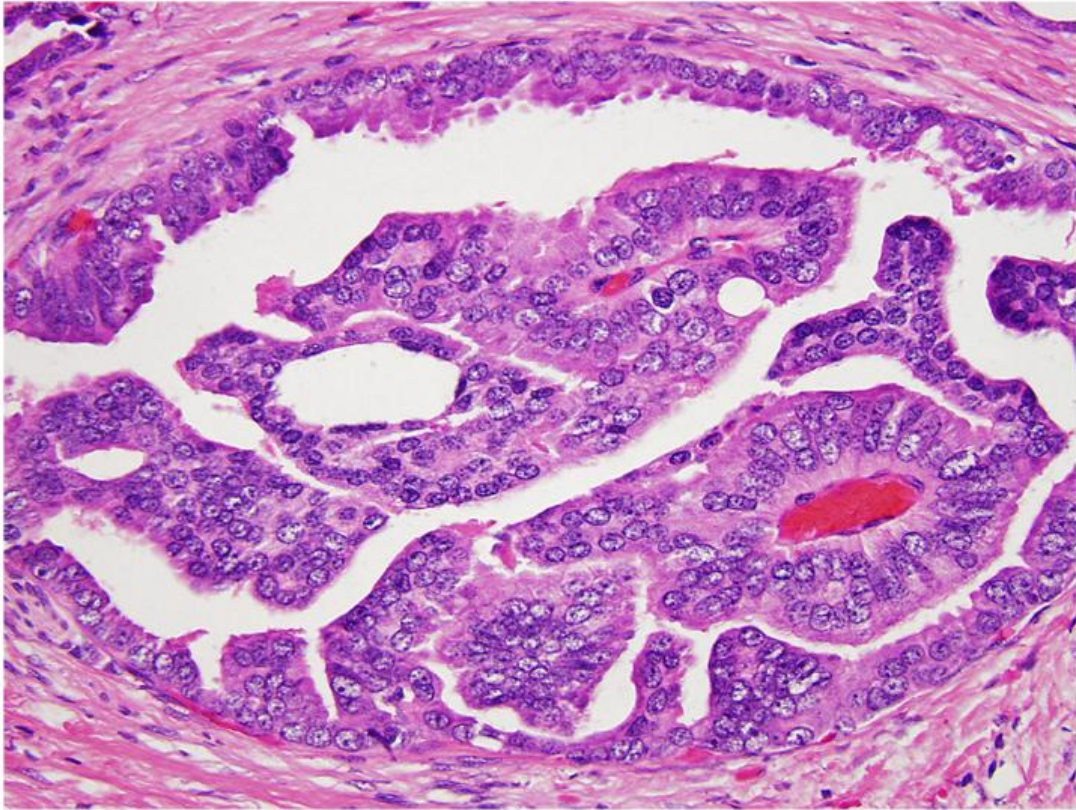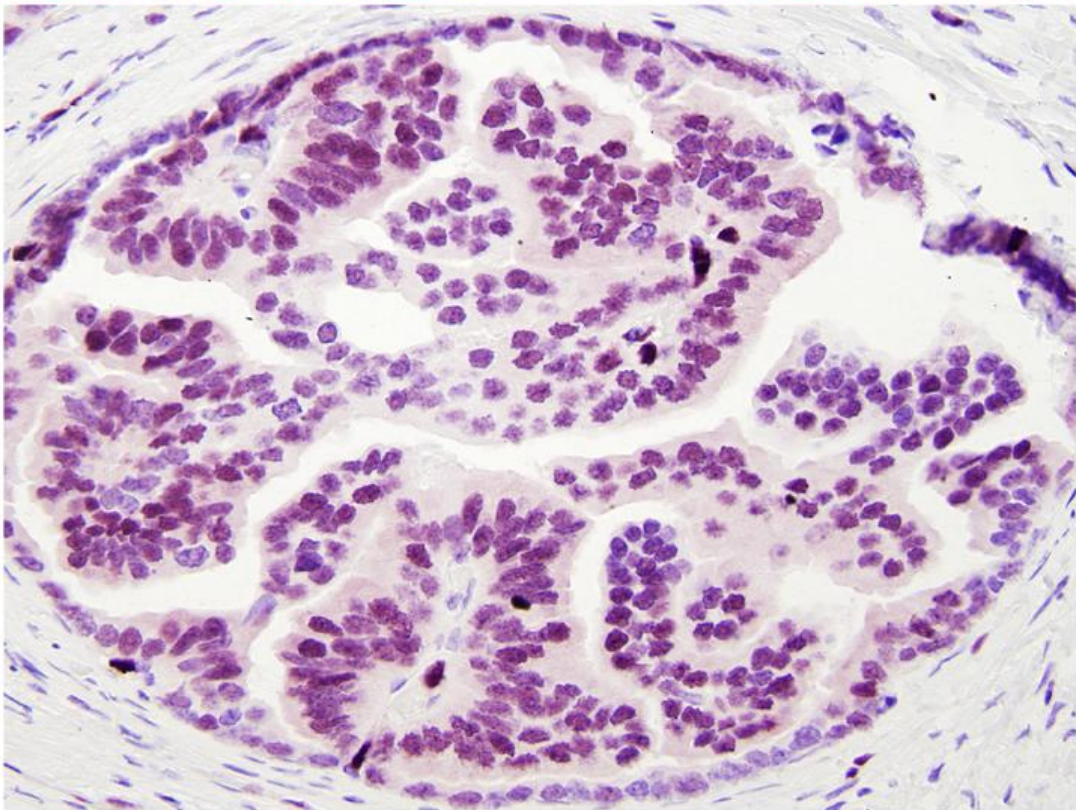
Supplementary Figure 5

e

f

g

h

Supplementary Figure 5. ERG oncoprotein expression in sub-types of prostatic adenocarcinoma. Foamy gland carcinoma: H&E stain (a) and ERG-MAb IHC (b). Mucinous prostatic adenocarcinoma: H&E (c) and ERG-MAb IHC (d). ERG positivity was noted predominantly in higher grade nuclei. Ductal carcinoma: H&E (e) and ERG-MAb IHC (f). Tumor cells are negative, reactivity to ERG-MAb is restricted to endothelial cells (f). Ductal carcinoma: H&E (g) and ERG-MAb IHC (h). Tumor cells and endothelial cells are positive for ERG-MAb IHC (h).

Supplementary Figure 6
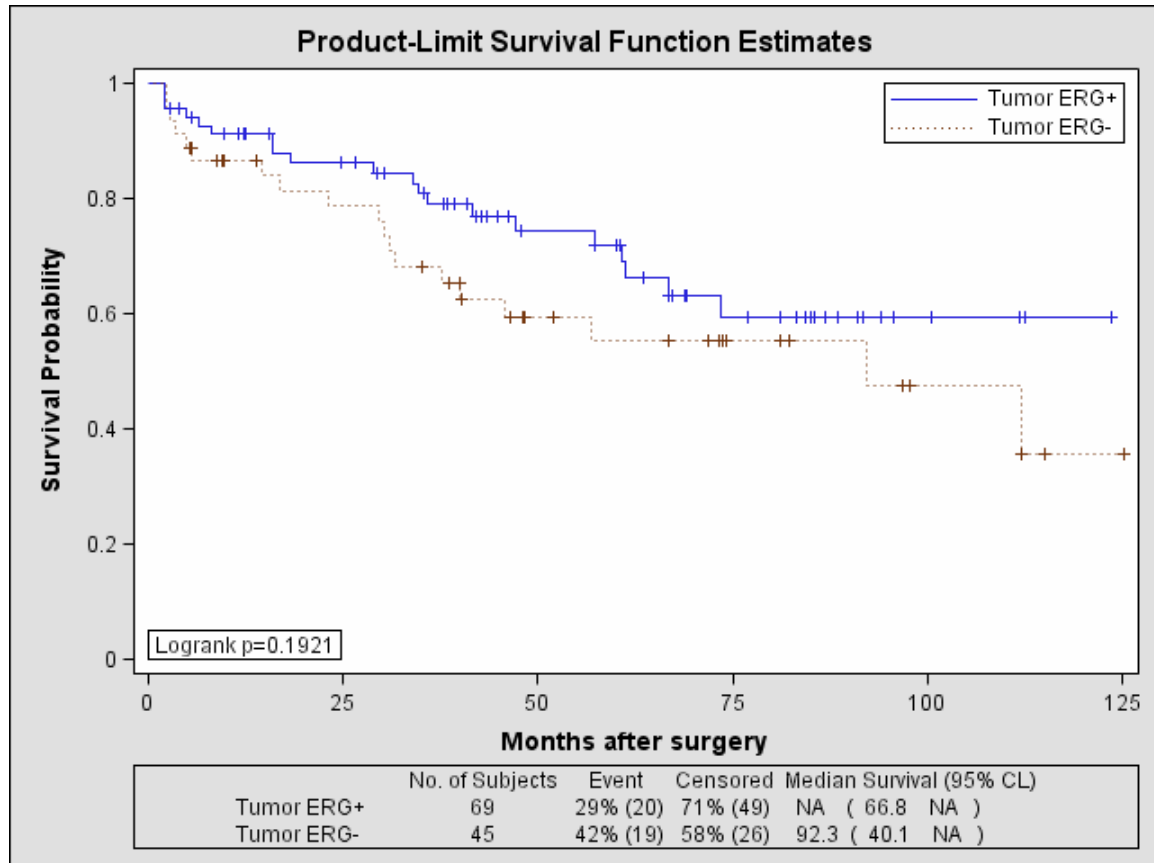
Supplementary Figure 6. Kaplan-Meier PSA recurrence-free survival curve for patients with ERG oncoprotein status. ERG positive and ERG negative status of prostatectomy specimens was evaluated for association with PSA recurrence after radical prostatectomy. PSA recurrence was defined as two consecutive serum PSA read-outs above or equal to 0.2 ng/ml.

Supplementary Table 1

a

Variables	n	%
Race		
Caucasian	105	79.6
African American	27	20.4
Pathological T stage		
pT2	34	25.8
pT3-4	84	63.6
pT2r1(x) [1]	14	10.6
Prostatectomy Specimen-Gleason Score [2]		
6	33	25.6
7	59	45.7
8 to 10	37	28.7
Margin status		
Negative	70	59.3
Positive	48	40.7
Tumor grade [3]		
Gleason pattern 3 (Well diff.)	160	61.3
Gleason pattern 4/5 (Moderately/Poorly diff)	101	38.7
Biochemical recurrence [4]		
No	76	65.5
Yes	40	34.5

b

Variables	n	Mean $\pm$ SD	Median (range)
Age at surgery (year)	132	61.1 $\pm$ 7.4	62.3 (40.2-75.2)
Pretreatment PSA ng/ml)	132	7.7 $\pm$ 4.8	6.4 (1.1-31.4)
Total tumor volume (cc)	132	10.4 $\pm$ 9.4	7.2 (0.03-52.8)
Follow up months after surgery	130	66.5 $\pm$ 35.6	67.6 (2.7-159.3)

Supplementary Table 1. Clinico-pathological features of prostate cancer patients. (a) Demographics and clinico-pathological features (categorical) of prostate cancer patients. [1]Data from patients with pT2r1(x) tumors were not used in this analysis. [2]Corresponds to Gleason score of the index tumor represented in the section except for three patients (Case number 83, 112 and 132). [3]Histological appearance in the observed areas. [4]Two consecutive PSAs $\geq$ 0.2 ng/ml. (b) Demographics and clinico-pathological features (continuous) of prostate cancer patients.

Supplementary materials and methods

Immunoblot assay for analysis of ERG protein products in HEK-293 cells transfected with wt ERG or TMPRSS2-ERG expression vectors
HEK-293 (ATCC, #CRL-1573) cells were grown in DMEM medium, supplemented with 10% fetal bovine serum and 2 mM glutamine. Cells ($2x10^6$) were seeded onto 10 cm dishes and maintained for two days, before transfection with 4 µg of expression vector plasmid DNAs. Lipofectamine 2000 (Invitrogen, Cat# 11668-027) was used for transfection, and 48 hours later cells were harvested and ERG protein was analyzed by Western blot by using the ERG-MAb at 1:5000 dilution.

Detection of ERG oncoprotein in human tumor cell lines
VCaP cells (ATCC, #CRL-2876) were grown in DMEM medium (ATCC, #30-2002). COLO 320 (ATCC, #CCL-220.1), MOLT4 (ATCC, #CRL-1582) and LNCaP cells (ATCC, #CRL-1740) were grown in RPMI-1640 medium (ATCC, #30-2001). KG1 cells (ATCC, #CCL-246) were grown in Iscove's Modified Dulbecco's Medium (ATCC, Cat# 30-2005). Cell culture media were supplemented with 10% fetal bovine serum (ATCC, #30-2020) and 2 mM glutamine. Approximately $10x10^6$ cells were harvested, lysed in M-PER mammalian protein extraction buffer (Pierce, Cat#78501) and the protein concentration were determined by Bradford assay (Biorad, Cat#500-0006). Fifty µg of total cell lysates were separated on NuPAGE Bis-Tris (4-12%) gels and wt ERG or ERG protein encoded by *TMPRSS2-ERG* were analyzed by Western blot using ERG-MAb at 1:5000 dilution.

Evaluation of TMPRSS2-ERG fusion status with dual-color interphase FISH
FISH break-apart assay as described by Perner *et al.,* [4] was used to analyze the *ERG* rearrangement on chromosome 21q22.2. The assay utilized a biotin-14-dCTP labeled BAC clone RP11-24A11 (reacted with red avidin-rhodamine) and a digoxigenin-dUTP-labeled BAC clone RP11-137J13 (reacted with green fluorescein-tagged anti-DIG antibody), complementary to centromeric and telomeric regions adjacent to the ERG locus, respectively. BAC clones were kind gifts from Dr. Settara C. Chandrasekharappa, National Human Genome Research Institute, and the probes were prepared by GenePro Diagnostics, Inc. (Rockville, Maryland). Probes were applied onto tissues in hybridization assays and analyzed by fluorescence microscopy.

Analysis of ERG mRNA by branched-chain DNA (bDNA) signal amplification
For bDNA analysis areas identified as tumors were marked, removed by scraping and homogenized in 600 µl Tissue Homogenization Solution (THS) followed by the addition of 12 µl (50 µg/ml) proteinase K. Specimens were incubated for 7 h at 65°C. The samples were centrifuged for 5 min at 24°C to pellet any debris. Supernatants were transferred to fresh microfuge tubes, avoiding any residual paraffin. All supernatants were analyzed immediately using the QuantiGene 2.0 Assay (Panomics, Fermont, CA). Each sample was assayed in duplicate. Eighty microliters (10 ng) of the homogenate were used for the amplification of *TMPRSS2-ERG* fusion type A [5] and 5 µl (0.625 ng) for housekeeping genes: *ACTB, B2M* and *RPL19*. In addition, VCaP mRNA was used as the positive control. To capture target RNAs, sample dilutions were prepared by combining

appropriate volumes of samples in THS. A working probe set for each target was prepared by combining 12 μl of the probe set with 40 μl of the blocking reagent (for target genes only) or 40 μl of nuclease-free water (for 28S RNA), lysis mixture and nuclease-free. The working probe set was mixed and kept at room temperature. To prepare the capture plate, 60 μl of each working probe set was transferred to assigned wells. Assay control and RNA samples were then made up to 100 μl with Hybridization Working Reagent (HWR) and hybridized overnight at 55°C. The plates were washed three times with wash buffer. Preamplifier was then added followed by amplifier, labeled probe and finally the chemi-luminescence substrate with washes after incubation in each reagent. The read-outs were measured in Modulus Luminometer.

Statistical analysis
Frequencies were calculated for categorical patient clinico-pathological features while measures of central tendency (mean and median) and dispersion (standard deviation and range) were assessed for continuous patient features. Biochemical recurrence was defined as two consecutive serum PSA readings ≥ 0.2 ng/ml after 2 months post-radical prostatectomy. Kaplan-Meier survival analysis and log rank test were used to examine the difference of biochemical recurrence-free survivals across tumor ERG oncoprotein status groups (positive vs. negative). *P* value of 0.05 was adopted as statistically significant. The SAS version 9.2 was used for all data analysis.

Supplementary References

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3. Owczarek CM, Portbury KJ, Hardy MP, O'Leary DA, Kudoh J, Shibuya K *et al*. Detailed mapping of the ERG-ETS2 interval of human chromosome 21 and comparison with the region of conserved synteny on mouse chromosome 16. *Gene* 2004; **324:** 65-77.
4. Perner S, Demichelis F, Beroukhim R, Schmidt FH, Mosquera JM, Setlur S *et al*. TMPRSS2:ERG fusion-associated deletions provide insight into the heterogeneity of prostate cancer. *Cancer Res* 2006; **66**(17)**:** 8337-8341.
5. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW *et al*. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science* 2005; **310**(5748)**:** 644-648.